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AWARD NUMBER: DAMD17-99-1-9499

TITLE: Cell Motility and Invasiveness of Neurofibromin-Deficient Neural Crest Cells and Malignant Triton Tumor Lines

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REPORT DATE: June 2006

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-06-2006	Final Addendum	1 Jun 2005 – 31 May 2006

4. TITLE AND SUBTITLE

Cell Motility and Invasiveness of Neurofibromin-Deficient Neural Crest Cells and Malignant Triton Tumor Lines

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-99-1-9499

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Texas Health Sciences
Center at San Antonio
San Antonio, Texas 78229-3900

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our purpose is to examine the role of the NF1 gene product, neurofibromin, in modulating the migratory and invasive properties of neural crest cells (NCC) and neural crest-derived sarcoma cells. As a negative regulator of Ras signaling, neurofibromin may influence the responses of NC-derived cells to growth factors and extracellular matrix (ECM) molecules that affect motility. We have completed our analyses of Nf1-/- embryonic NCC invasiveness in vitro, compared effects of neurofibromin deficiency in different embryonic mesenchymal cell populations derived from cranial and trunk regions, and characterized expression of neural stem cell markers in the cell populations used. We have completed immunoblot analyses of PDGF signaling in sarcoma lines derived from cisNf1+/-;p53+/- mice. Finally, we have expanded our analyses of the cisNf1+/-;p53+/- mouse model to characterize mutant frequency in normal and tumor tissues, and to compare DNA repair capacities in neurofibromin-deficient Schwann cells and Schwann cell-derived tumor cell lines.

15. SUBJECT TERMS
NF1, neural crest cells, cell migration, malignant peripheral nerve sheath tumor, p53, genomic instability, DNA repair, neurofibromin, Schwann cell

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	10	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION

For a number of normal and neoplastic cell types, loss or diminution of the Nf1 gene product, neurofibromin, leads to changes in migratory behavior, in particular invasiveness. These alterations in cell motility potentially affect the establishment and growth of neurofibromas and café-au-lait macules, metastasis of malignant peripheral nerve sheath tumors (MPNST), and the development of subtle central nervous system abnormalities that could contribute to learning disorders in NF1 patients. The main objectives are to characterize the changes in cell motility for neurofibromin-deficient embryonic neural crest cells, and to identify environmental cues that influence the invasiveness of MPNST cell lines derived from spontaneous tumors in *cisNf1*+/-;*p53*+/- mice. Over the past year, we completed our experiments to compare *Nf1*+/+, +/-, and -/- neural crest cell migration through fibronectin and laminin matrices, and to characterize platelet-derived growth factor (PDGF) and PDGF receptor signaling pathways that influence proliferation and migration of MPNST cell lines. In addition, we have continued to develop and expand a new direction, examining genomic instability and DNA repair capacity, for the cisNf1+/-p53+/- mouse model and the cell lines generated from tumors, and for neural crest-derived cells isolated from Nf1-deficient mouse embryos.

BODY

Spontaneous mutant frequency in tumors and normal tissues of cisNf1;p53 mice

See Appendix (manuscript in review at *Mutation Research*)

cisNf1;p53 sarcoma lines and TGFβ/ PDGF signaling (revised SOW, Tasks 3 and 4)

To begin to identify changes in growth factor responsiveness that may lead to malignant transformation of plexiform neurofibromas, and may account for differences in proliferative capacity and invasiveness between our neural crest-derived sarcoma lines, we have examined signaling pathways activated by TGF-β and PDGF-BB. Last year, we reported on the heterogeneity in responsiveness to PDGF-BB among our different MPNST lines, particularly in the activation of the PDGF receptor; we have also found that production of the PDGF ligand varies between cell lines, raising the possibility that some MPNST may exhibit autocrine stimulation of PDGF signaling pathways. Dang and DeVries (2005) reported abnormal responses to PDGF-BB in human MPNST, as compared to normal Schwann cells, and we have used our MPNST lines and cultures of embryonic Schwann cells to examine this signaling pathway in the context of Nf1-deficient murine tumor cells and Schwann cells.

Invasiveness of Nf1-neural crest cells (revised SOW, Task 2)

Several different classes of signaling molecules, distributed along migration routes and within localization sites, influence the motility, proliferation, survival, and differentiation of neural crest-derived cells throughout development. Many of these environmental cues directly or indirectly affect early aspects of NCC behavior by signaling through receptor tyrosine kinases. Neurofibromin, a GAP encoded by the neurofibromatosis type 1 (Nf1) gene, has a crucial role as a negative regulator of RTK signaling through Ras in neural crest-derived cells. Mouse embryos that lack neurofibromin (*Nf1*-/-) die before embryonic day 14 (E14: Brannan et al., 1994; Jacks et al., 1994); however, many neural crest-derived cell types can be isolated prior to this stage and maintained in culture. Sensory and sympathetic neurons isolated from Nf1-deficient mouse embryos survive and extend neurites in the absence of neurotrophins required by wild-type cells, presumably due to consitutive Ras activation (Vogel et al., 1995, 2000). Loss of neurofibromin in Schwann cells leads to accelerated differentiation (Kim et al., 1995), unless hyperproliferation is induced by increasing cyclic AMP levels (Kim et al., 1997). To characterize the effects of *Nf1* gene dosage on the motility of neural crest-derived cells, we isolated first branchial arch mesenchymal populations, as well as trigeminal ganglion non-neuronal cells, from mouse embryos and measured their performance in transwell invasiveness assays. In agreement with results reported for other cell types, we find that neurofibromin deficiency significantly increases the invasive potential of cranial neural crest populations in vitro.

In last years's report, we summarized results of experiments that demonstrate that loss of neurofibromin affects the invasiveness of neural crest-derived (trigeminal ganglion) and cranial mesenchymal (branchial arch) cell populations, but not the invasiveness of trunk mesodermal (limb) cells at the stages examined. In addition, a comparison of the invasiveness of Nf1-/- trigeminal and branchial arch cells between E10 and E12 indicates that the roles of neurofibromin in controlling motility may become increasingly important as development proceeds. We showed that the PI3-kinase inhibitor, LY294002, attenuates the increased migration of *Nf1-/-* trigeminal

neural crest cells; we have also decreased invasive capacity by treating these cells with the MAP kinase inhibitors PD98059 and U0126 (data not shown). These data are consistent with results using neurofibromin-deficient Schwann cells derived from E12.5 dorsal root ganglia (Huang et al., 2004). In order to increase the likelihood that these data will be published, we have added two sets of experiments over the past year: 1) characterization of expression of neural crest markers in the cell populations used (trigeminal neural crest, branchial arch mesenchymal, limb bud mesenchymal), 2) additional inhibitor studies with both the embryonic cell populations and the sarcoma cell lines. We are completing these experiments, and will include the data in the revised manuscript.

Chromodomain protein MRG15 as a modifier of the cisNf1;p53 phenotype

Over the past two years, we have characterized the effects of haploinsufficiency for a transcriptional regulator, MRG15, on tumor latency, cell proliferation, and genomic instability, in the context of the cisNf1+/-;p53+/- mouse model for NF1-related sarcomas. To begin to identify possible modifiers of the malignant NF1-associated tumor phenotype, we combined our cis*Nf1*;*p53* mutations with a targeted null mutation in the *Mrg15* gene. *Mrg15* encodes a chromodomain protein that is present in complexes involved in transcriptional activation (Pardo et al., 2002). *Mrg15*-/- mouse embryos typically die around E14.5, and exhibit defects in the forebrain, heart, and lungs; in addition, *Mrg15*-/- mouse embryonic fibroblasts exhibit a proliferation defect (Tominaga et al., 2005). Because MRG15 protein derepresses the *B-myb* promoter by association with retinoblastoma protein, thus promoting cell cycle progression, we reasoned that a reduction in Mrg15 might affect tumor latency and cell proliferation characteristics in *cisNf1*+/-;*p53*+/- mice.

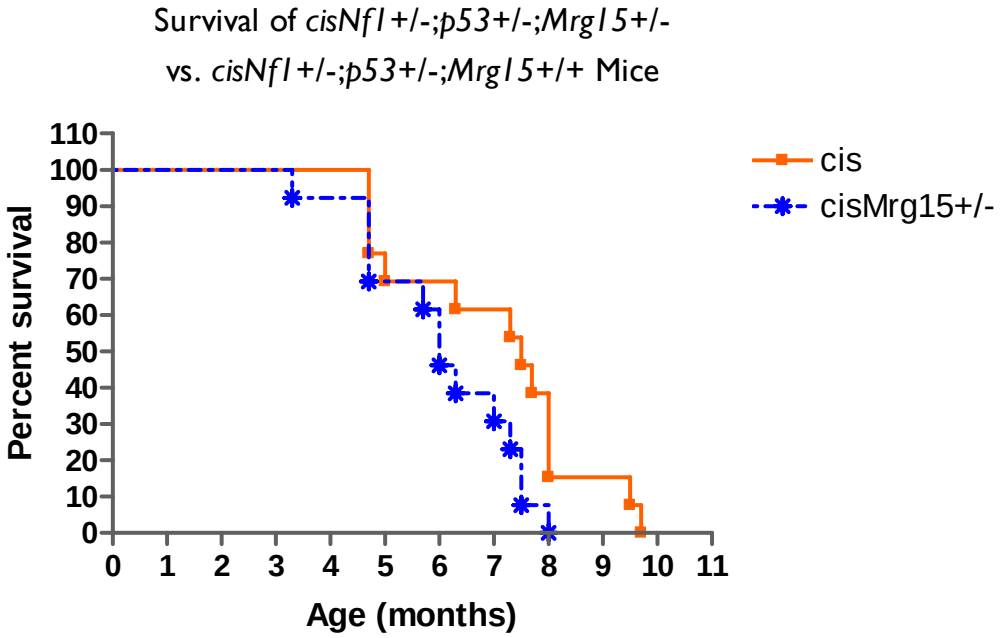

Figure 1. Kaplan-Meier Survival Analysis. For each group, n= 13, and the logrank test indicates a slightly significant difference between the two curves (P=0.0462).

Figure 1 shows that there is a slight acceleration of tumor latency in mice that are haploinsufficient for Mrg15; no differences in tumor spectrum were observed.

Through its ability to interact with histone acetyltransferase (HAT) complexes, MRG15 is also involved in DNA damage repair pathways. Drosophila Mrg15 is part of a multiprotein dTip60 chromatin-remodeling complex, involved in repair of DNA double-strand breaks (DSB), and depletion of dMrg15 in cells or embryos

leads to defective repair following gamma-irradiation (Kusch et al., 2004). To determine whether MRG15 levels influence repair of DSB in the context of murine neural crest-derived sarcomas, we used the comet assay to compare DNA repair capacity, following gamma-irradiation, in lines derived from cisNf1+/-;p53+/-; Mrg15+/- and cisNf1+/-;p53+/-;Mrg15+/+ littermates. All cell lines had undergone LOH for both Nf1 and p53 (data not shown). **Figure 2** shows that, although sarcoma lines from Mrg15+/+ mice have repaired almost 100% of the DNA damage by 90 minutes, the Mrg15 haploinsufficient lines exhibit only 30-60% repair at this time point.

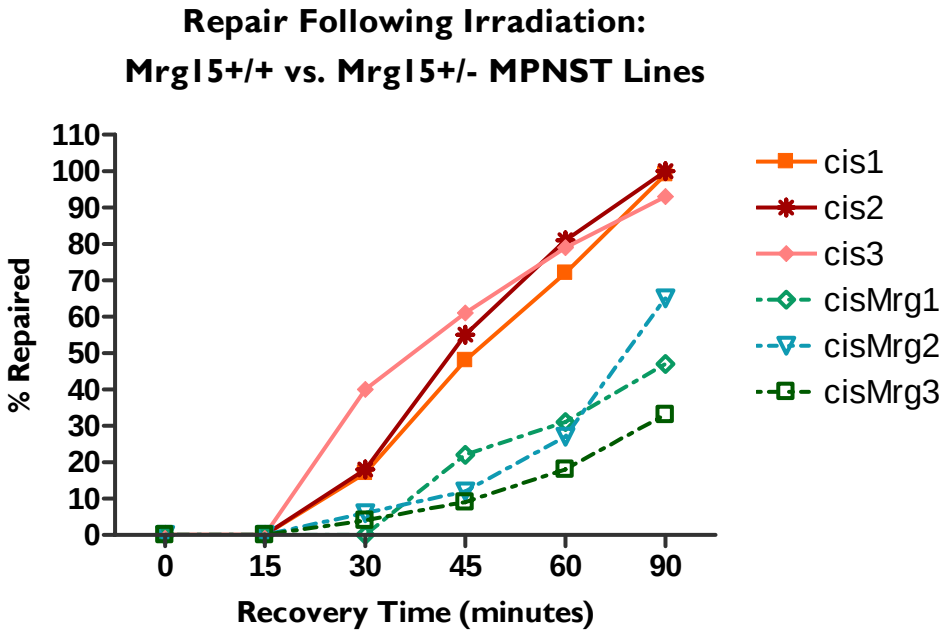

Figure 2. Deficient repair of DSB by Mrg15+/- cisNf1;p53 sarcoma cell lines. Nine to eleven Gy of gamma radiation was used to induce DNA double-strand breaks, and cells were allowed to recover for different periods of time, followed by analysis of DNA damage in individual nuclei using the comet assay (single-cell gel electrophoresis). At least 500 cells were scored for each cell line and time point.

Tables 1 and 2 summarize the repair data, following gamma-irradiation, for a number of sarcoma cell lines, as well as for normal Schwann cells derived from E13.5 Nf1+/- mouse embryos.

Table 1. Repair of DSB, sarcoma lines from cisNf1+/-;p53+/-;Mrg15+/+ mice

	% Repair (Time in Minutes)					
Cell Line ID (passage #)	**0'**	**15'**	**30'**	**45'**	**60'**	**90'**
Tu25-5 (P25)	0	0	2	42	68	88
Tu26-4 (P30)	0	0	0	16	28	48
cis023.2 (P11)	0	0	3	18	45	96
Tu70-7 (P18)	0	0	40	61	79	93
Tu9-10 (P17)	0	0	18	55	81	100
Tu8-7 (P19)	0	0	17	48	72	99
cis002.A3 (P15)	0	0	6	29	44	85
cis002.A3 (P16)	0	0	17	25	37	83
cis002.A2 (P15)	0	0	12	38	45	93
cis002.A2 (P17)	0	0	15	28	56	91
cis002.A5 (P10)	0	0	8	23	59	98

cis002.A5 (P12)	0	0	6	26	56	97
E12.5 Nf1+/- Schwann cells	0	0	16	50	69	94
E12.5 Nf1+/- Schwann cells	0	0	22	42	76	95

Table 2. Repair of DSB, sarcoma lines from cisNf1+/-;p53+/-;Mrg15+/- mice

	% Repair (Time in Minutes)					
Cell Line ID (passage #)	**0'**	**15'**	**30'**	**45'**	**60'**	**90'**
cisMRG014.A3 (P10)	0	0	0	22	31	47
cisMRG005.A1 (P10)	0	0	6	12	27	65
cisMRG005.A1 (P12)	0	0	5	17	35	48
cisMRG012.A6 (P12)	0	0	4	9	18	33
cisMRG001.B12 (P7)	0	0	0	10	27	37
cisMRG001.B12 (P8)	0	0	0	9	24	33
cisMRG005.A5 (P11)	0	0	0	9	15	33
cisMRG005.A5 (P13)	0	0	0	26	37	51
cisMRG011.6 (P15)	0	0	0	2	14	27
cisMRG011.6 (P18)	0	0	0	ND	21	32
cisMRG011.6 (P20)	0	0	0	18	27	53
cisMRG005.A7 (P11)	0	0	0	6	26	46
cisMRG005.A7 (P13)	0	0	0	8	17	38
cisMRG011.8 (P15)	0	0	0	17	26	43
cisMRG011.8 (P18)	0	0	0	26	36	38
cisMRG005.A2 (P13)	0	0	0	ND	13	24
cisMRG005.A2 (P15)	0	0	ND	3	ND	27

To determine whether Mrg15-deficient sarcoma cells can complete repair, given a longer time course, we used the comet assay to measure repair at 120 minutes following gamma radiation**. Figure 3** shows that some Mrg15+/- lines are simply delayed in repair, whereas others exhibit a substantial amount of unrepaired DNA even at this later time point. Our recent results indicate that clonal cell lines derived from the same tumor (e.g. cisMRG011.6 and cisMRG011.8) exhibit similar DNA repair capacities.

Our DNA repair results with Mrg15+/- murine sarcoma lines are consistent with those obtained by our collaborators, K. Tominaga and O. Perreira-Smith, using Mrg15-deficient (+/- and -/-) mouse embryo fibroblasts. Currently, we are comparing cloning efficiency following gamma irradiation, as well as MRG15 protein levels and the nuclear distribution of proteins involved in DNA damage recognition and repair, in sarcoma lines derived from both cisNf1+/-;p53+/-; Mrg15+/- and cisNf1+/-;p53+/-;Mrg15+/+ mice, and preparing a manuscript to be submitted to *Oncogene*.

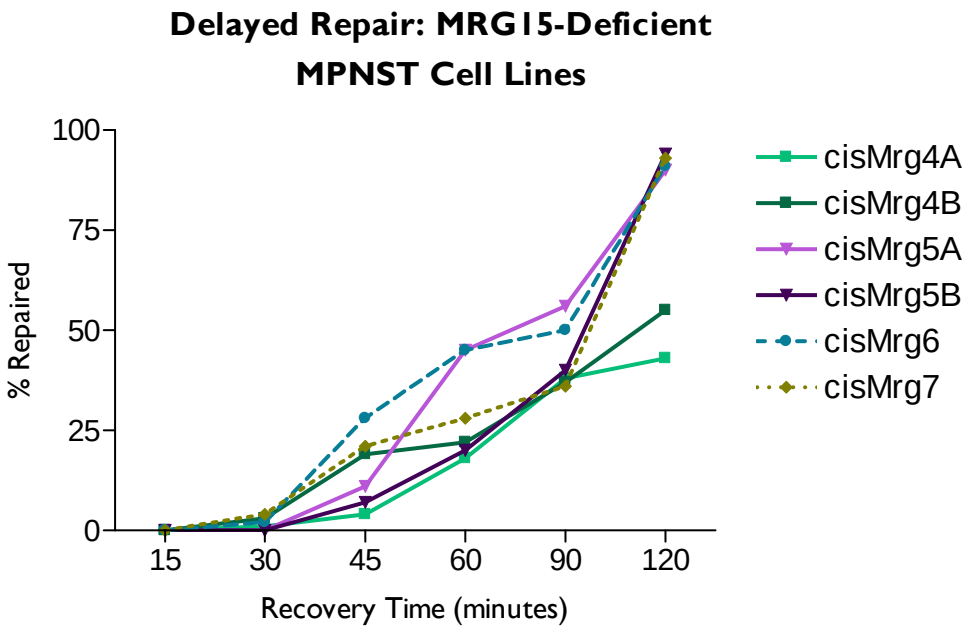

Figure 3. Some Mrg15-deficient sarcoma lines complete repair by 120 minutes following gamma-irradiation. DSB repair data were obtained as described for Figure 2. At least 500 cells were scored for each cell line and time point.

KEY RESEARCH ACCOMPLISHMENTS

- Completed assessing the effects of PI3 kinase, Akt, and MAP kinase inhibitors on Nf1-/- NCC invasiveness
- Completed analyses of expression of neural stem cell and neural crest derivative markers in population of cranial neural crest cells isolated from Nf1-/-, +/-, and +/+ embryos at different stages (E10-E12)
- Completed inhibitor studies for signaling pathways activated by TGF-beta and PDGF-BB in MPNST-like sarcoma cell lines isolated from cisNf1+/-;p53+/- mice
- Completed analyses of Mrg15 gene dosage effects on tumor latency and tumor spectrum in cisNf1+/-;p53+/- mice
- Adapted comet assay (single-cell gel electrophoresis) to examine DNA double-strand break repair in neurofibromin-deficient Schwann cells and MPNST lines
- Manuscript on spontaneous mutant frequency in cisNf1+/-;p53+/- mouse tumors and tissues in review, *Mutation Research* (avaialble to DoD upon request)

REPORTABLE OUTCOMES

Manuscripts and Abstracts

- June 2003: Molecular Biology of NF1 and NF2 Meeting, oral presentation. "Mutant frequencies in NF1 tumors and Nf1+/-;Trp53+/- tissues"
- June 2003: Molecular Biology of NF1 and NF2 Meeting, poster presentation. "Invasiveness of neurofibromin-deficient cranial mesenchymal cells"
- June 2005: Children's Tumor Foundation, Molecular Biology of NF1, NF2, and Schwannomatosis Meeting, poster presentation. "A mild mutator phenotype arises in NF1-associated malignancies"
- June 2006: Children's Tumor Foundation, Molecular Biology of NF1, NF2, and Schwannomatosis Meeting, platform presentation. "DNA repair capacity in Schwann cells and sarcoma lines isolated from Nf1-deficient mice"

- Miller, S.J., Li, H., Rizvi, T.A., Huang, Y., Johansson, G., Bowersock, J., Sidani, A., Vitullo, J., **Vogel, K.S.**, Parysek, L.M., DeClue, J.E., and Ratner, N. (2003) Brain lipid binding protein in axon-Schwann cell interactions and peripheral nerve tumorigenesis. Molecular and Cellular Biology 23, 2213-2224
- Ling, B.C., Wu, J., Miller, S.J., Monk, K.R., Shamekh, R., Rizvi, T.A., Decourten-Myers, G., **Vogel, K.S.**, DeClue, J.E., Ratner, N. (2005) Role for the epidermal growth factor receptor in neurofibromatosis-related peripheral nerve tumorigenesis. Cancer Cell 7, 65-75
- Garza, R., Hudson, R.W., Walter, C.A., and **Vogel, K.S.** (in review) A mild mutator phenotype arises in malignancies associated with neurofibromatosis type 1. (*Mutation Research: Fundamental and Molecular Mechanisms of Carcinogenesis*)
- White, C., and **Vogel, K.S.** (revised manuscript in preparation) Increased invasiveness of neurofibromin-deficient branchial arch mesenchymal and trigeminal ganglion neural crest cells. (*Experimental Cell Research*)
- Hudson, R.W., and **Vogel, K.S.** (in preparation) PDGF signaling in murine Nf1- and p53-deficient sarcoma cell lines.
- Tominaga, K., Perreira-Smith, O., and **Vogel, K.S.** (in preparation). Mrg15 haploinsufficiency reduces DNA double-strand break repair capacity in a mouse model for MPNST.

Cell Lines and Animal Models

- cisNf1+/-;p53+/-; lacI+ mice and sarcoma cell lines, for quantitation of mutant frequency and characterization of genomic instability
- cisNf1+/-;p53+/-;Mrg15+/- mice, for analyses of chromotin-modifying complexes and genomic instability in neural crest-derived sarcomas
- 30 novel sarcoma cell lines derived from cisNf1+/-;p53+/-;Mrg15+/- mice, for analyses of DNA repair capacity following gamma-irradiation and treatment with other genotoxic agents

Funding Obtained and Pending

- San Antonio Area Foundation: "Mutant Frequency in a Mouse Tumor Model for NF1" $11, 815
- San Antonio Cancer Institute: " Nf1 and Mrg15 Regulation of Tumorigenesis and Neural Cell Proliferation" $15,000
- Nathan Shock Center on Longevity and Aging Studies, “DNA Damage and Repair in Nf1-Deficient Schwann Cells throughout the Lifespan”, Pending
- USMRMC NFRP, “Rapid Assessment of DNA Repair Capacity in *Nf1*-deficient Schwann Cells and Astrocytes”, Pending

Employment and Training Opportunities

- Rene Garza, Research Assistant
- Robert Hudson III, Senior Research Assistant (currently first-year dental student at UTHSCSA)

CONCLUSIONS

First, we have demonstrated that neurofibromin deficiency alters the motility and invasiveness of cranial neural crest and mesenchymal cell populations in early embryogenesis; loss of neurofibromin does not, however, appear to alter the invasiveness of limb-derived (mesodermal) mesenchymal cells. This is likely to reflect the variable importance and expression levels of different RasGAPs in distinct cell lineages throughout development. We would like to begin to understand how Nf1 gene expression is regulated both temporally and spatially during embryonic development and differentiation, particularly in neural crest lineages, and we have begun to examine this using real-time quantitative PCR.

Second, we have demonstrated that perturbations in levels of a gene, Mrg15, involved in chromatin remodeling, can dramatically alter DNA repair capacity following genotoxic damage, in the context of murine neural crest-derived sarcoma cells. Mrg15 haploinsuuficiency had a modest effect on tumor latency in the cisNf1+/-;p53+/- mouse model for NF1 malignancies.

"So what" section Over the past few years, it has become increasingly clear that stem cells persist in a variety of adult tissues, and may in fact represent a cell population that is particularly prone to malignant transformation. NF1 is primarily a disorder of the neural crest, and therefore it is of potential interest to

understand the roles of neurofibromin in regulating the behavior of these cells. Our experiments address the role of neurofibromin in modulating neural crest cell responsiveness to environmental cues, in the context of motility. Phenotypic characterization of the sarcomas that arise in cisNf1+/-;p53+/- mice is consistent with a neural crest stem cell origin for murine MPNST-like tumors, i.e. a variety of differentiated traits can be expressed by a given tumor cell.

In the past year, we have adapted a rapid, inexpensive assay, single-cell gel electrophoresis or "comet", for use with murine Schwann cells and MPNST lines, to examine sensitivity to genotoxic agents, as well as DNA repair capacity. DNA repair capacity has been suggested to contribute to the variable expressivity of NF1 in humans (Wiest et al., 2003), and Nf1+/- mice are more susceptible to development of therapy-induced malignancies following genotoxic treatment (Chao et al., 2005). We feel that analyses of genotoxin sensitivity and DNA repair capacity in normal and neoplastic Schwann cells in NF1 patients may have predictive value for lifetime tumor burden, and may indicate potential risks of genotoxic treatments.

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